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A Panel Study on Epigenetics, Markers of Oxidative Stress, and Lung Function among Children with Respiratory Disease Exposed to Industrial Air Pollution

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preface

Epidemiological and not Statistical

Applied and not Theoretical

- Study description [aim, design]
- Simple data analysis [standard approach]
- Graphical chain model
- Direct and indirect effect

Study description: objective

To study DNA methylation of IL-6 and iNOS promoters, DNA methylation of ALU and LINE-1 repetitive elements and their association with markers of bronchial inflammation (Fractional exhaled Nitric Oxide: FeNO), lung function (FEV-1) and wheezing episodes in children with respiratory symptoms exposed to industrial air pollution,

a panel study nested in a cross-sectional investigation on respiratory symptoms in school-children resident in the high environmental risk area of Milazzo – Valle del Mela (Sicily, Italy) was conducted in the period December 2007 – April 2008.

In the Milazzo-Valle del Mela area are located a major petrochemical plant and a oil-powered thermal plant.

Study description: design - 1

- The cross-sectional phase was conducted during April-May 2007 on all 2506 resident children attending the local primary schools. Respiratory health was recorded by the ISAAC questionnaire administered to the parents for a total of 2244 children (89.5% completeness).

  Using the answer at the items on diagnosis of asthma, wheezing symptoms in the last 12 months (three items), chest tightness in the last 12 months, nocturnal dry cough in the last 12 months and use of bronchodilators in the last 12 months,

  50 children were selected, whose had asthma diagnosis, wheezing symptoms and positives to minor symptoms or use of medications. Parents of 39 children (78%) gave assent to participate.

Study description: design - 2

- The study sample was divided in ten small groups

  Each group was followed-up for seven days, starting on Saturday and ending on Friday the week after. The study started on December 8th, 2007 and ended on April 18th, 2008.

- A weekly diary on respiratory symptoms and drugs (ATS derived) and a daily diary on hourly activities of the child and indoor sources of pollution were recorded by the parents. (WHO)
- Expiratory flow (**FEV-1**) was recorded on the morning and afternoon (between 4 – 6 o' clock pm). We used PiKo®-1 FEV-1 Meter. The maneuver was supervised by the nurse on the afternoon.
- Fractional exhaled Nitric Oxide concentration **FeNO** (NIOX analyzer Aerocrine) (ATS/ERS) were measured by the nurse in the afternoon. Ambient NO concentrations were also taken.
- On day fourth and seventh of follow-up the child went to the out-patient clinic to execute **nasal brushing** by trainee personnel.

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10 – 12 mar.	14 mar.	15 - 21 mar.	18 mar 21 mar	Milazzo L. Rizzo Carrubbaro Bastione Baronello	Mar Par La S Cat Par
22 - 24 mar.	25 mar.	26 mar. - 2 apr.	28 mar 1 apr.	Milazzo S. Cuore Garibaldi	Par Fur Ball Buc Car
7 – 9 apr.	11 apr.	12-18 Apr.	15 apr. 18 apr.	Pace elem. Pace Media	Car Mar Fun Pirr Sal...

- **Nasal Mucosa Cell Collection and DNA Extraction**

  Each of the children was first asked to blow his/her nose to clear any mucous discharge. The nasal cavity was washed with saline solution (0.9% NaCl). Nasal cells were collected by soft brushing with a Cytobrush® . The maneuver was executed twice per child, day fourth the right nostril, day seventh the left.

  Nasal cells were gently rinsed with saline solution (0.9% NaCl and 10% acetylcysteine), incubated for 30 min at room temperature (100 cycles/min). Cell pellets were frozen. DNA was extracted using the Wizard Genomic DNA purification kit (Promega, Madison, WI).

- **DNA Methylation Analysis**

  We used highly-quantitative analysis by means of PCR-pyrosequencing on bisulfite-treated DNA (Tarantini et al EHP 2009) to measure DNA **methylation in repetitive elements Alu/LINE-1**, and promoter regions IL-6 and iNOS. 0.5 µg DNA (concentration 50 ng/µL) was treated using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA).

- We developed the assay for ***IL-6 promoter* methylation** by locating the *IL-6* promoter using the Genomatix Software (Genomatix Software Inc, Ann Arbor, MI, USA) on chromosome 7. A 50-µL PCR was carried out in 25 µL GoTaq Green Master mix (Promega), 10 pmol forward primer, 10 pmol reverse primer, 50 ng bisulfite-treated genomic DNA, and water.
- Measuring ***iNOS* promoter methylation** is in Tarantini et al. (EHP 2009)
- For all assays we used built-in controls to verify bisulfite conversion efficiency.

- Compared with other common methods of DNA methylation analysis, pyrosequencing-based assays have the advantage of producing individual measures of methylation at more than one CpG dinucleotide, thus reflecting more accurately DNA methylation in the region.

- In the Alu or LINE-1 assays, we measured the percentage of 5mC (%5mC) at each of three CpG dinucleotide positions that are repeated over the human genome with the sequence of interest.
- In the *iNOS* promoter assay, we measured %5mC at each of two individual CpG dinucleotides within a CpG island located in the gene promoter;
- In the *IL-6* promoter assay, we measured %5mC at two individual CpG dinucleotides within a CpG island located downstream in the proximity of the gene promoter.

exposure characterization

- Personal measurements of air pollutants concentrations were taken on one witness per group of children. The groups were assembled to be homogeneous by school attendance (the same classroom) and area of residence. One of the children served as witness. She wore passive dosimeters for nitric dioxide (NO2) and sulphur dioxide (SO2) (Passam-Laboratory) and bore a portable instrument for PM2.5 measurement (Sidepack AM510).

  Eight calibrations were made againts a gravimetric standard (AirFlow HS Avantech) by the EPA of Tuscany.

- Ambient air pollutants concentrations were measured between November 2007 and April 2008 on 21 locations in the schoolyards of the primary and secondary schools of the study area by means of passive dosimeters (Passam Laboratory). NO2, SO2 and benzene, toluene and xylenes were measured over a week, twice per month. PM2.5 daily concentrations were measured by the gravimetric monitor located at a baricentric schoolyard on 100 days between December 2007 and April 2008.

Simple data analysis:
methylation markers as predictors of FEV-1 FeNO

- **FEV-1**. We fitted a GEE population average model, Gamma family with log link, exchangeable correlation structure, robust estimator of standard error
- **FeNO** (restricted to measurements - 69.3% - at ambient NO< 6ppb). We fitted a left censored regression model on log-transformed response variable, detection limit 4ppb (5.7%), robust estimator of se.

We **adjusted** for day of the week, parental education, subject's height and weight (when appropriate), gender, passive smoking, mould or dump in the house, traffic intensity, recent respiratory infections, use of steroids for asthma, temperature, relative umidity

Sensitivity analysis was performed using two jackknife approaches to standard error estimate, random effect and AR models, a linear model for independent observations.

results: subjects characteristics

- The enrolled children were on average
  8.86 yr old (sd=0.79; min 8 - max 11),
  138.4 cm (sd=7.1) tall,
  weighted 38.1 kg (sd=9.9).
  38% of them were females.

  Piko-1® FEV1 was on average 2.13 l (sd=0.31).

  6.8 measurements per children were available (afternoon).

  FEV1% (based on spirometry) was: <90 25.6%; 90-99 30.8%; 100+ 43.6% .

  Mean FeNO was 34.9 ppb (sd=30.3)

  5.7% below the instrument detection limit of 4ppb, 4.5 meas. per ch.

  All the children had asthma and wheezing during exercise, dry cough, cough and phlegma. 52% had one course of inhaled steroids, 30% chest tightness, 27% nocturnal or diurnal wheezing.

results: risk factors

74.4% at least one parent had 14+ yrs of **education**

10.3% **mould or dampness** in child's room during his/her 1st yr life

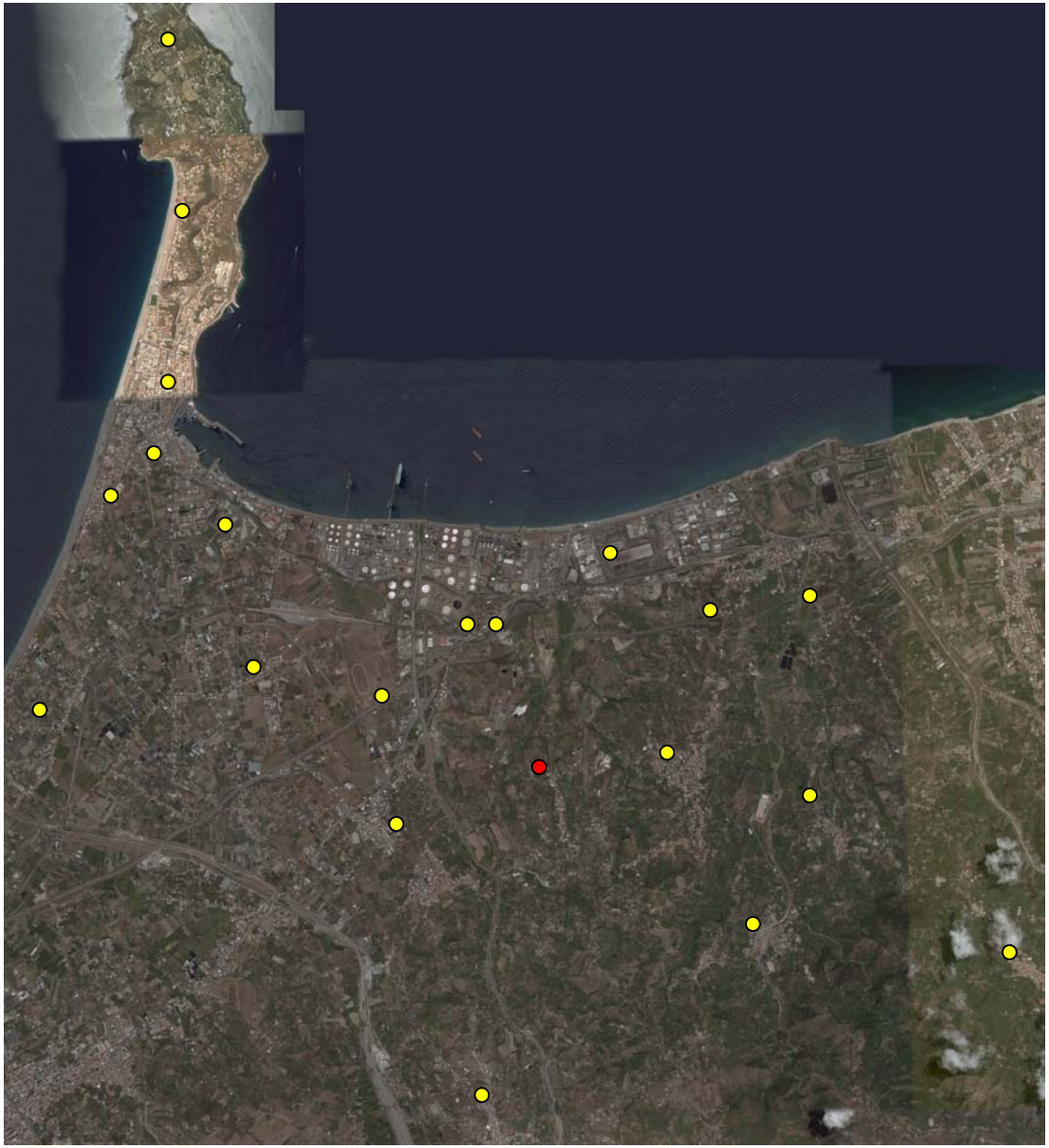
23% of the mothers were **current smokers**
64% were **never smoker**

61% close to streets with high vehicular **traffic**

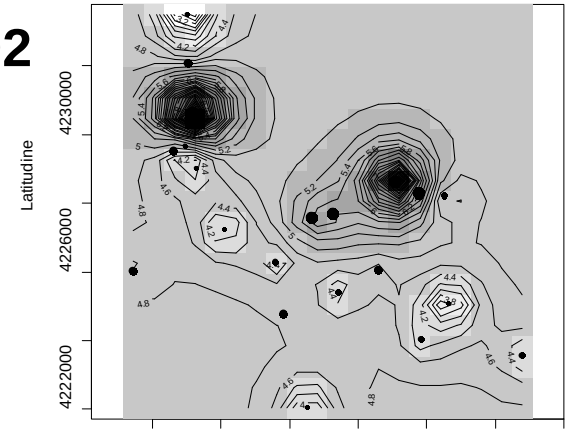
Ambient air pollutants concentrations of PM2.5 were 23.3 $\mu g/m^3$ (median 21.3, 90° percentile 40.0 and max 77.3).

NO2 was above 40 $\mu g/m^3$ weekly averages only in one location.

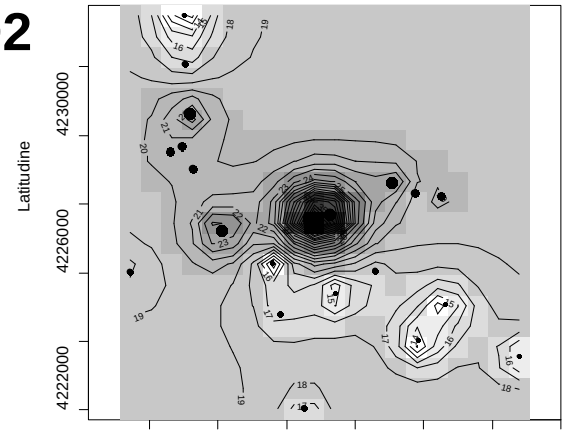
SO2 was above 20 $\mu g/m^3$ weekly average in the proximity of the plants, consistently with prevalent north-south wind directions. Hourly excesses of SO2 above 100 $\mu g/m^3$ were documented in three locations, by the Sicilian EPA, Department of Messina.



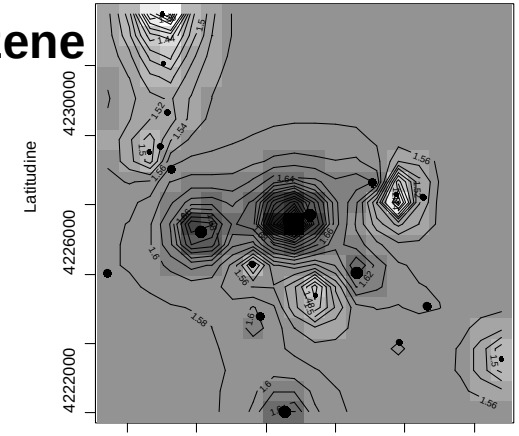
SO2



NO2



Benzene



Bayesian kriging of passive dosimeters data on 21 locations

results: descriptive statistics

fev1		fev1	alu	line	il6	inos
> 2.12 l	mean	2.56	23.20	72.70	**52.77**	63.21
	sd	0.37	0.68	4.35	**14.01**	7.79
	n	37	37	37	**37**	37
< 2.12 l	mean	1.82	23.14	72.03	**48.67**	64.60
	sd	0.20	1.08	4.09	**10.89**	8.90
	n	37	37	37	**37**	37
Total	mean	2.19	23.17	72.36	**50.71**	63.90
	sd	0.47	0.90	4.20	**12.63**	8.33
	n	74	74	74	**74**	74

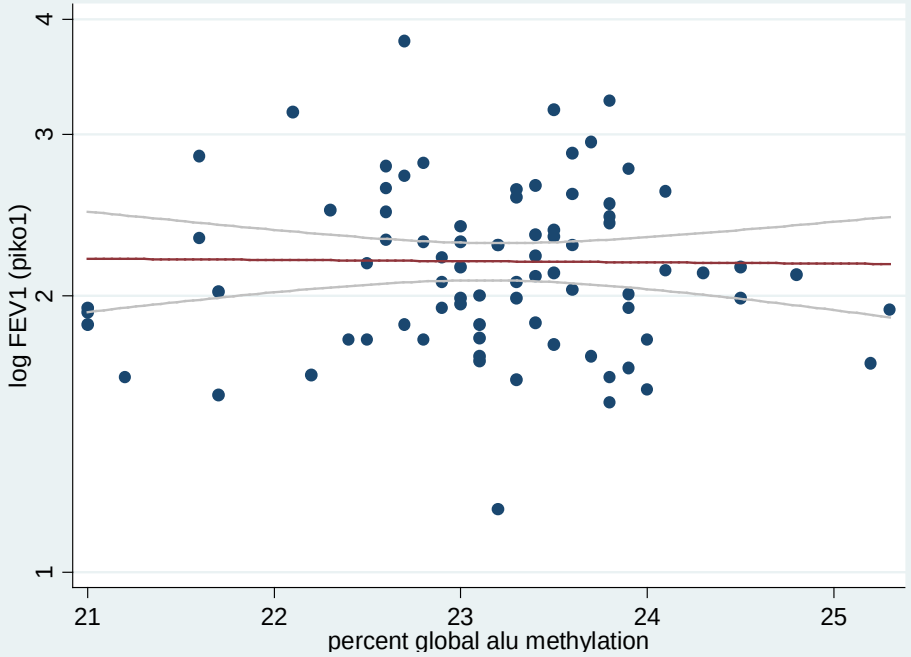
DNA methylation of IL-6 promoter appeared to be hypomethylated when FEV-1 was below the median
48.67 % vs 52.77 %

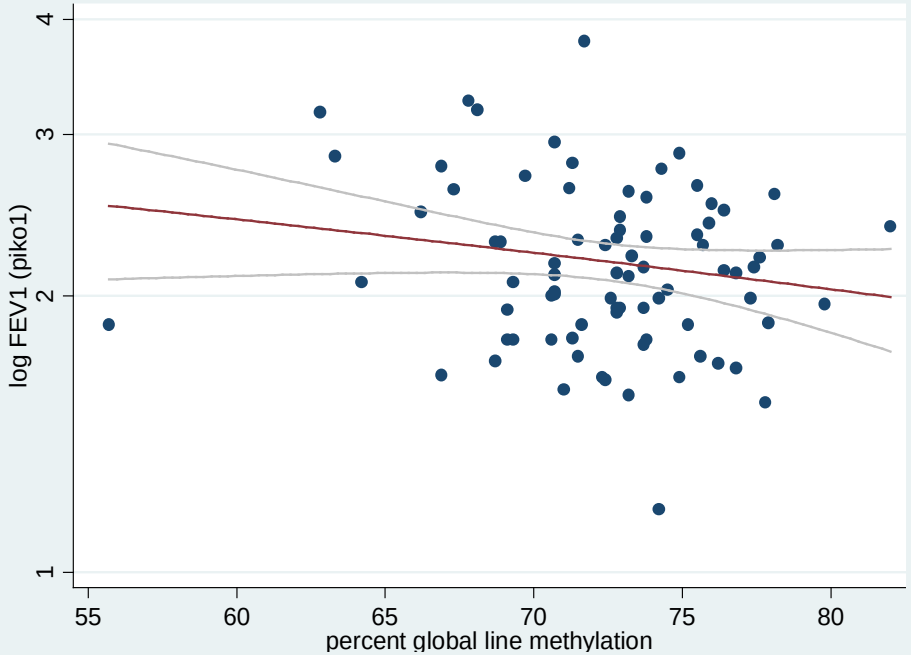
number of valid measures

feno		log-feno	alu	line	il6	inos
< 24 ppb	mean	2.42	23.20	72.55	**52.68**	**66.26**
	sd	0.47	0.77	4.81	**14.79**	**7.01**
	n	38	38	38	**38**	**38**
> 24 ppb	mean	3.95	23.13	72.17	**48.65**	**61.42**
	sd	0.47	1.03	3.50	**9.68**	**8.97**
	n	36	36	36	**36**	**36**
Total	mean	3.16	23.17	72.36	**50.71**	**63.90**
	sd	0.90	0.90	4.20	**12.63**	**8.33**
	n	74	74	74	**74**	**74**

DNA methylation of IL-6 and iNOS promoters appeared to be hypomethylated when FeNO was above the median

IL-6: 48.65 % vs 52.68 %
iNOS: 61.42 % vs 66.26 %


log FEV1 (piko1)
1
2
3
4
21
22
23
24
25
percent global alu methylation


log FEV1 (piko1)
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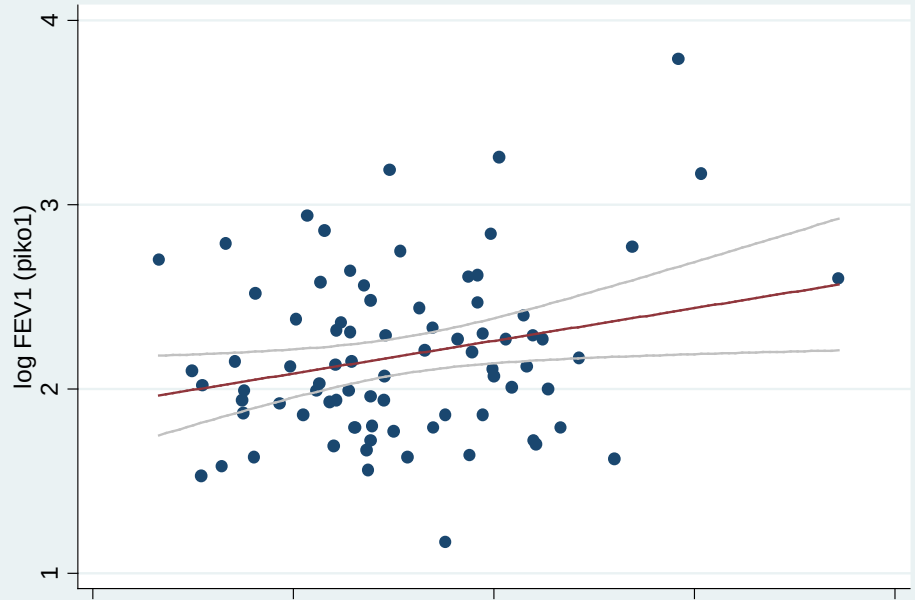
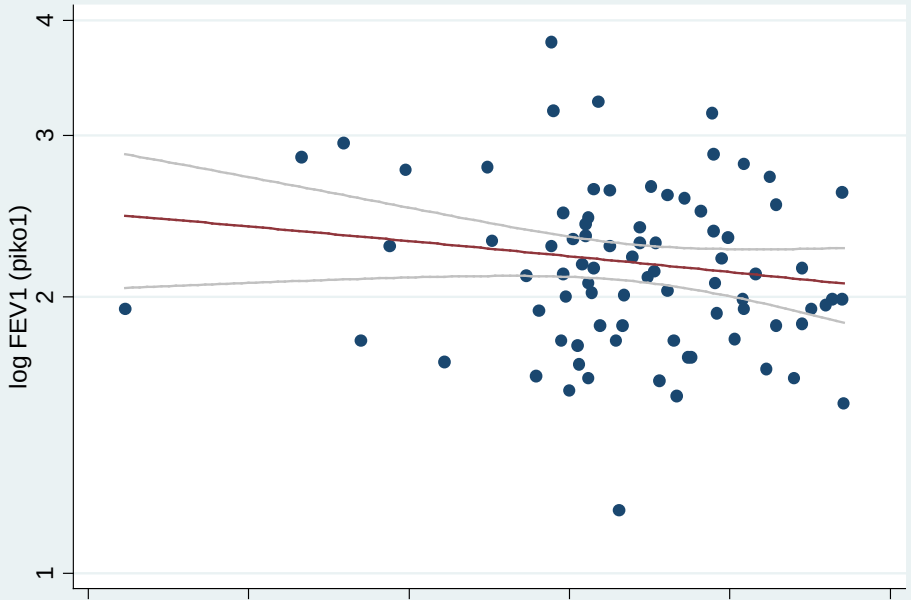
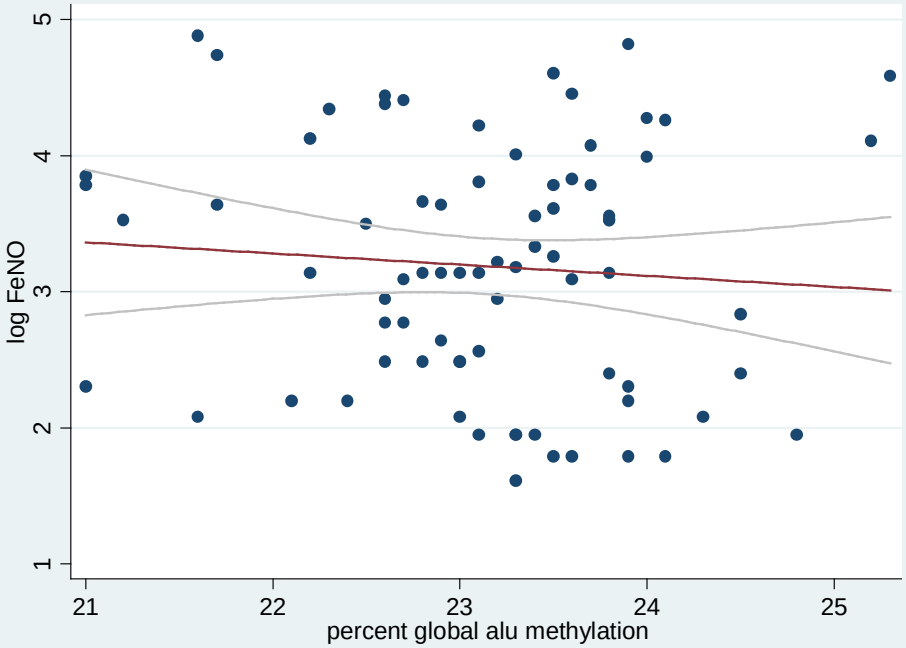
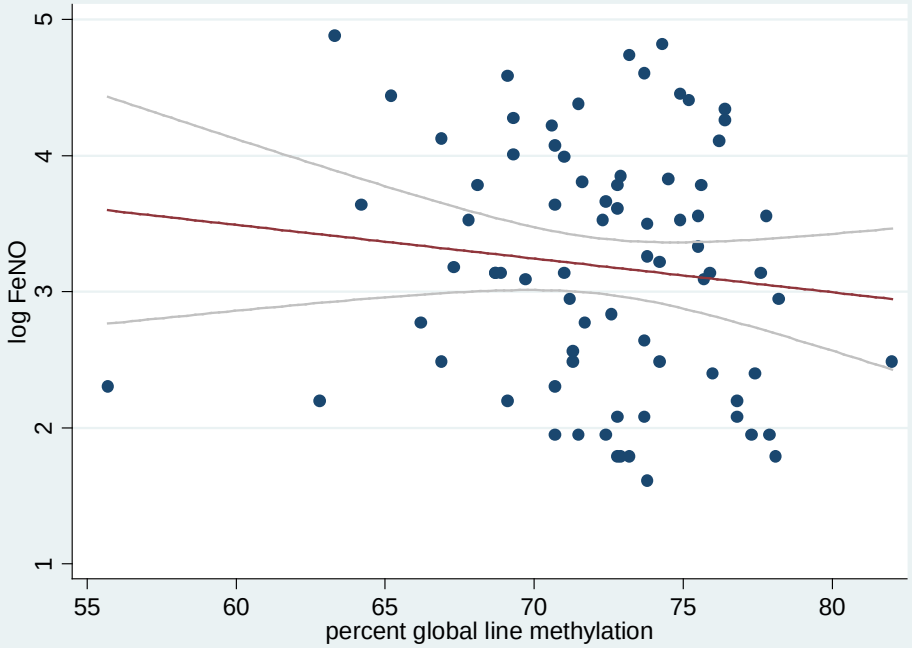
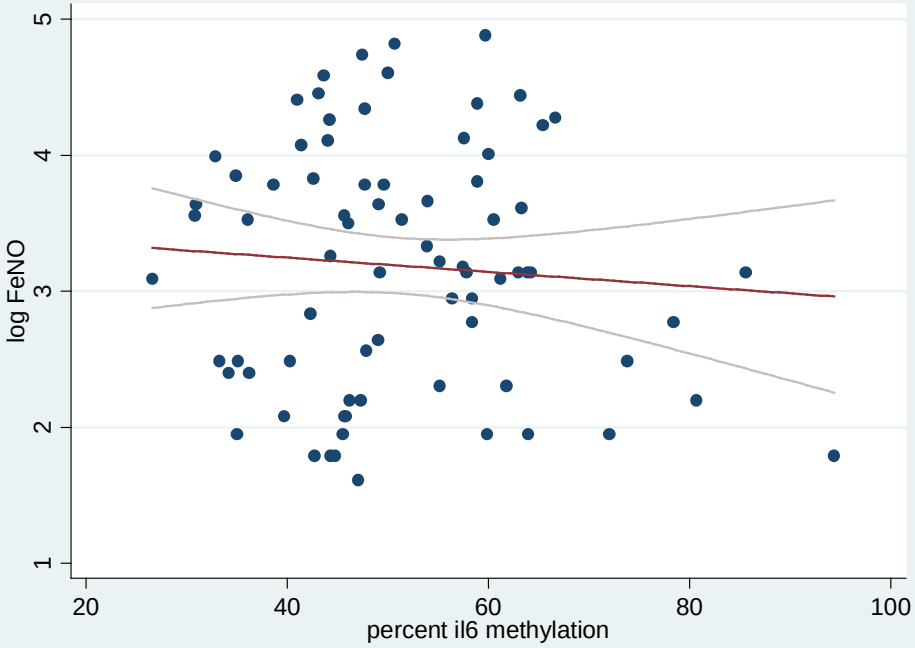
2
3
4
55
60
65
70
75
80
percent global line methylation

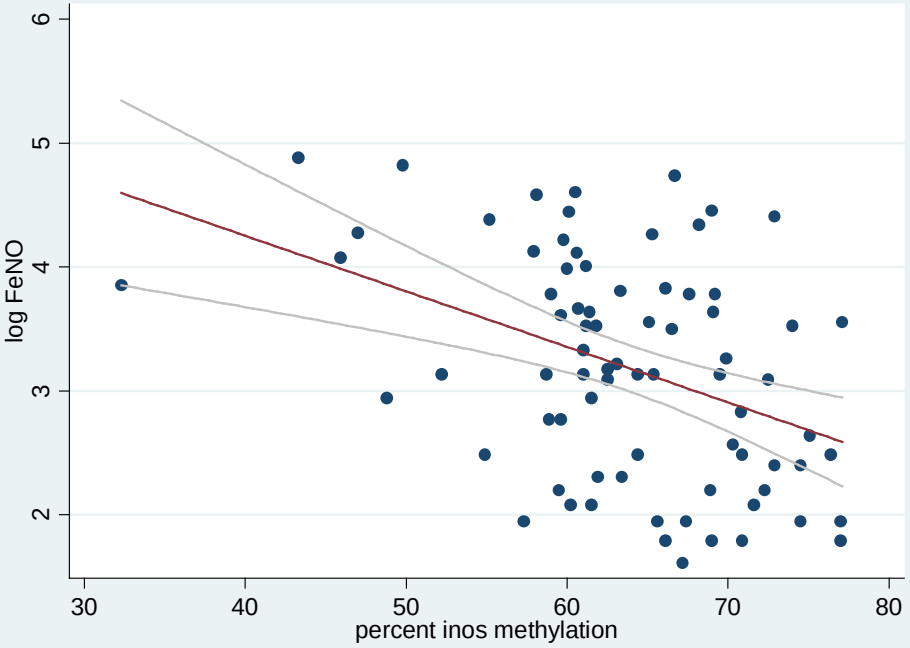

log FEV1 (piko1)
1
2
3
4


log FEV1 (piko1)
1
2
3
4


log FeNO
1
2
3
4
5
21
22
23
24
25
percent global alu methylation


log FeNO
1
2
3
4
5
55
60
65
70
75
80
percent global line methylation


log FeNO
1
2
3
4
5
20
40
60
80
100
percent il6 methylation


log FeNO
2
3
4
5
6
30
40
50
60
70
80
percent inos methylation

results: simple statistical models

- The GEE regression analysis of **FEV-1** on DNA methylation markers showed that **Hypomethylation of IL-6 promoter was associated to a decrease in FEV-1** (a reduction of 15.8% of methylation – the interquartile range – was associated with a reduction in FEV-1 of 5.2%, 90% CI 0.1; 10.6).

- The left-censored tobit regression analysis of **FeNO** on DNA methylation markers showed that **Hypomethylation of iNOS promoter was associated to an increase in FeNO**. In particular, a reduction of 9.2% of methylation – the interquartile range – was associated with an increase in FeNO of 66.2%, 90% CI 54.7; 80.1).

- Considering simultaneously the four DNA methylation markers and the two responses, FEV-1 and FeNO, the association between methylation of LINE-1 repetitive elements and FEV-1 disappeared while the association of methylation of the IL-6 and iNOS promoter with FeNO was more pronounced.

Multiple Regression analysis of FEV-1 (GEE model) and FeNO (lef-censored Tobit regression) on DNA methylation of IL-6 and iNOS promoters, and in Alu and LINE-1 repetitive elements. Backward step-wise model selection. Number of valid observation 74.

Step-1	FEV-1				FeNO			
	β	r.se.	90% CI	pvalue	β	r. se.	90% CI	pvalue
ALU	0.005	0.026	-0.038; 0.049	0.836	0.012	0.140	-0.221; 0.244	0.934
LINE	-0.005	0.007	-0.017; 0.08	0.534	-0.007	0.029	-0.055; 0.042	0.822
IL6	0.003	0.003	-0.02; 0.007	0.319	-0.014	0.006	-0.025; -0.004	0.019
iNOS	-0.003	0.003	-0.008; 0.001	0.224	-0.050	0.014	-0.073; -0.027	0.001

Final	FEV-1				FeNO			
	β	r.se.	90% CI	pvalue	β	r. se.	90% CI	pvalue
ALU								
LINE								
IL6	0.004	0.002	0.0001; 0.008	0.095	-0.015	0.006	-0.025; -0.006	0.009
iNOS					-0.050	0.013	-0.072; -0.027	<0.001

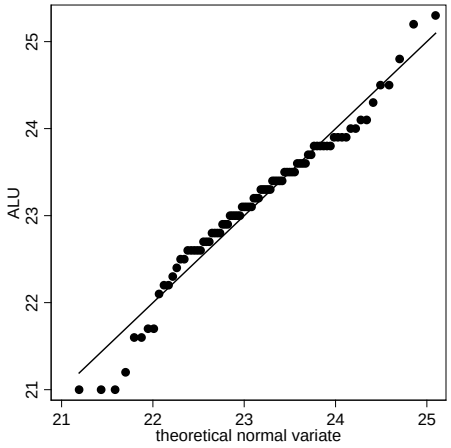
results: simple statistical models

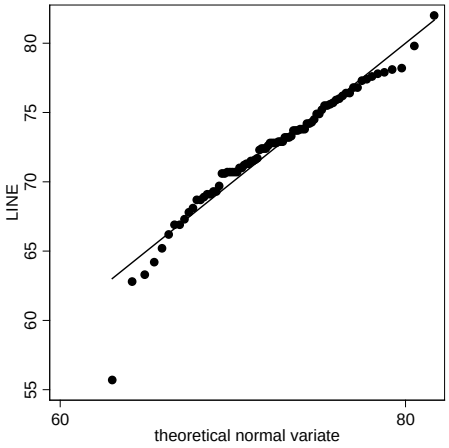
These findings were confirmed when analyzing the occurrence of **wheezing** treated as a binary variable (presence/absence): only methylation of IL-6 promoter appeared to be associated with symptoms (multiple logistic regression analysis: the odds ratio for the interquartile range of IL-6 promoter methylation of 17.2% was 0.40, 90% CI 0.17; 0.94).

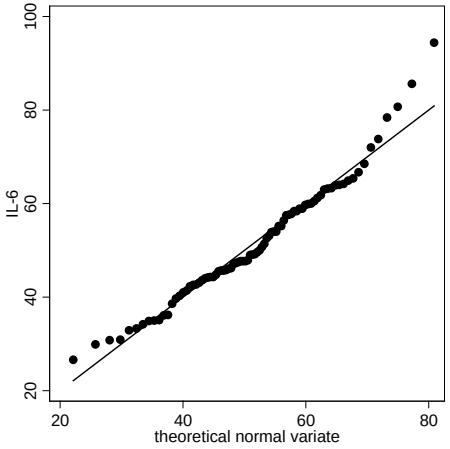
Wheezing		fev1	alu	line	**il6**	inos
No	mean	2.19	23.23	72.48	**52.00**	63.77
	n	59	62	62	**62**	62
Yes	mean	1.98	23.06	72.75	**44.71**	67.10
	n	12	12	12	**12**	12
Total	mean	2.15	23.17	72.36	**50.71**	63.90
	n	71	74	74	**74**	74

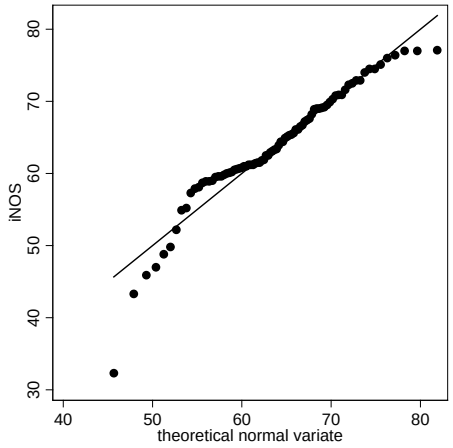
Graphical chain model

- DNA methylation is expressed as a percentage. However, the empirical distribution is symmetrical and roughly normally distributed. The statistical model used to evaluate associations between DNA methylation and clinical, subject's characteristics or environmental pollutants was a **Gaussian linear mixed model to take into account within and between subject source of variation.**
- To minimize arbitrariness in the model building phase, we decided in all the analyses, to **adjust for the same set of confounders without selection**.









Graphical chain model

- The analysis of multivariate dependencies can be solved by means of a series of separate regression analyses or can be described by modelling directly the concentration matrix. (Cox DR and Wermuth N. 1998)

- The idea is to represent the conditional relationships among variables. The results can be visualized as a graph whose vertexes represent variables and edges conditional relationships.

  Chain graphs are used when we can a priori partition the variables in explanatory, intermediate and responses. Each block contains a type of variables, edges within block represent conditional associations, between blocks directed edges (arrows) represent dependencies.

Graphical chain model

- We analysed jointly the four DNA methylation positions and their relationships with clinical outcomes.

  .
- In particular we modelled jointly ALU, LINE, IL-6,iNOS methylation positions as a set of explanatory variables, two markers (FEV-1 and FeNO) [as intermediate and presence of wheezing as outcome].

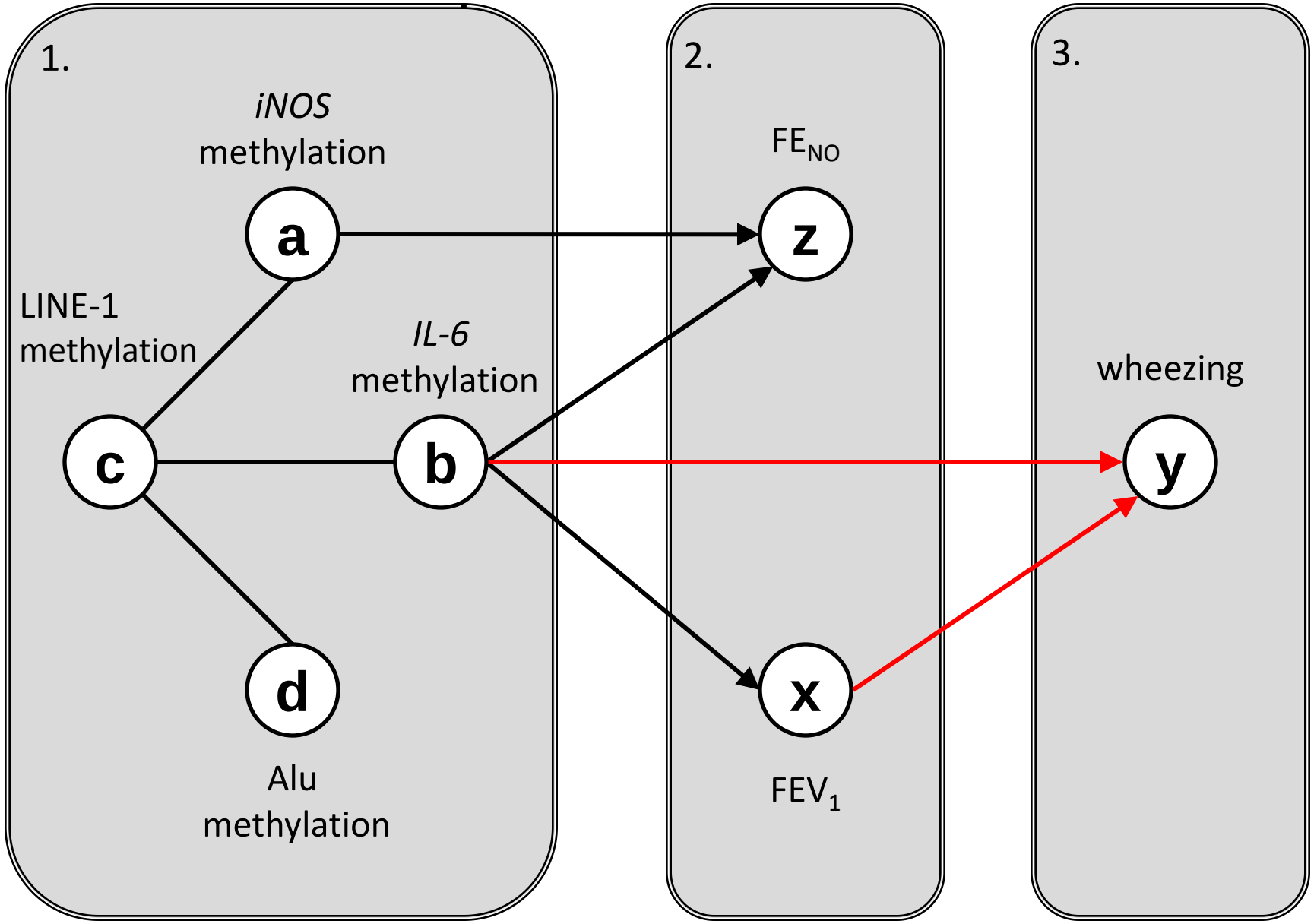
- Graphical chain models assume linearity and absence of second order interactions (effect modification) among continuous variables. This might be not realistic. Unobservable latent structures could also contribute to the observed association graph.

results: Graphical chain model

Global methylation indicators were correlated each other, while in our data DNA methylation of IL-6 promoter was inversely correlated to LINE-1 repetitive elements methylation.

Correlation coefficients (lower triangle) and partial correlation coefficients among DNA methylation of IL-6 and iNOS promoters, and in Alu and LINE-1 repetitive elements (in bold significant at α=0.05).

	ALU	LINE	IL-6	iNOS
ALU	1.0000	**0.3161**	0.1006	0.0830
LINE	**0.3337**	1.0000	**-0.3236**	0.2113
IL-6	-0.0387	**-0.3553**	1.0000	-0.1346
iNOS	0.1660	**0.3081**	-0.2227	1.0000


1.
2.
3.
iNOS
methylation
FE$_{NO}$
a
z
LINE-1
methylation
IL-6
methylation
wheezing
c
b
y
d
x
Alu
methylation
FEV$_1$

Direct and indirect effect

IL6 - FEV1 - WHEEZING

We follow VanderWeele (Epidemiology, 2009) and Robbins et al. (Epidemiology 2000) and fit a marginal structural model with stabilized weights.

The weights are proportional to the inverse probability of IL6 (“treatment”) and FEV-1 (“intermediate”).

The MSM is a regression of *Y (“response”* , here the binary variable wheezing) on *IL6* and *FEV-1 .* Confounding is controlled not by including covariates in the regression model but by weighting.

Direct and indirect effect

IL6 - FEV1 - WHEEZING

Two sets of weights are specified.
For each generic unit we specified the following weights:

$$w_{IL6} = \frac{\Pr(IL6)}{\Pr(IL6|\mathbf{C})}$$

$$w_{FEV1} = \frac{\Pr(FEV1|IL6)}{\Pr(FEV1|IL6,\mathbf{C},\mathbf{Z})}$$

The denominator of the w_{IL6} is the probability of having the IL6 methylation level the unit in fact had conditional on the covariates *C* taking the values *c*.

The denominator of the w_{FEV1} is the probability of having the value of the intermediate FEV1 that the unit in fact had conditional on IL6 and the covariates C,Z.

The numerator is used to stabilized the weights for efficiency reasons. Conditional densities are used for continuous variables.

Results:
direct effect of IL6 and FEV1 effect on wheezing

1. Fit a model for (IL6 | potential confounders): GLMM with confounders (weekday, age, sex, parental education, BMI, mother smoking status, dampness, traffic exposure, steroid medication, temperature and humidity)
2. Get the predicted values and compute stabilized weights (marginal density of IL6 in the numerator)
3. Fit a model for (FEV1 | the potential causes and confounders and IL6): GLMM with confounders and IL6 methylation marker
4. Get the predicted values and compute stabilized weights (the marginal density of FEV1 in the numerator)
5. Fit a MSM with IPW given by the product of the two weights.

wheezing	Odds Ratio	robust Std Err	90% CI
FEV1	0.21	0.22	0.04 ; 1.22
IL6	0.94	0.03	0.89 ; 0.98

discussion - 1

- Each child was assessed twice for DNA methylation markers, on the fourth and seventh day of follow-up.

  Associations between FEV-1 / FeNO and DNA methylation markers may be the results of subject's stable methylation status with severity of the disease, or short-term variation of clinical and methylation status.

  Two days lag could be too short time span.

  We found moderately low intraclass correlation coefficients (ALU r=0.20; LINE-1 0.10; IL-6 0.08; iNOS 0.43).

  The FEV-1 showed intraclass correlation coefficient of 0.47, FeNO 0.84 presence/absence of wheezing a weighted kappa of 0.79.

- We provide some evidence to the existence of short-term changes in DNA methylation and their association with asthma course and severity.

discussion - 2

- Promoter hypomethylation is usually associated with enhanced transcription of the gene.

  In our data suggests demethylation of their promoter regions provides independent contributions to the control of FeNO levels. Therefore our data suggest that increases in IL-6 expression may either increase *iNOS* expression, or cause post-transcriptional modifications.
- Moreover we showed that those changes in DNA methylation can be detected in nasal cell DNA.

- Lower methylation levels in the IL-6 promoter were moderately associated with airway obstruction (reflected by PIKO FEV1) as well as with wheezing.

  IL-6 expression has been consistently shown to be related to airway inflammation, and IL-6 increases have been previously shown to be tightly associated with decreases in FEV1.

discussion - 3

- Our study did not show independent association of DNA methylation in Alu / LINE-1 repetitive elements with FeNO, FEV-1 or wheezing.

  In our data, LINE-1 methylation was negatively associated with *IL-6* methylation and positively associated with *iNOS* promoter.

  This finding is consistent with previous reports.

  Our data indicate that the association of LINE-1 with health outcomes might just due to positive and/or negative association with gene promoters' methylation levels.

- Our study was based on analysis of DNA methylation using pyrosequencing, which is highly reproducible and accurate at quantifying DNA methylation (Bollati Cancer Res 2007; Yang NAR 2004).

Conclusion

- In this panel study of asthma children in Milazzo, Italy, we found that DNA methylation of the *iNOS* and *IL-6* genes in nasal cell DNA was associated with measures of airway inflammation and obstruction.

- In particular, in univariate regression models lower *iNOS* promoter methylation was associated with higher FeNO, whereas in multivariate regression lower methylation of both the *iNOS* and *IL-6* promoters were independently associated with higher FeNO. We also found that lower methylation of the *IL-6* promoter was associated with airway obstruction, as reflected in higher FEV-1 and wheezing, though both associations were estimated with large imprecision.

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